Plant Science, 59 (1989) 57 – 62
Elsevier Scientific Publishers Ireland Ltd.

POLYGALACTURONASE IN POLLEN FROM CORN AND OTHER GRASSES

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(Received April 8th, 1988)
(Revision received August 3rd, 1988)
(Accepted August 4th, 1988)

Polygalacturonase was found in pollen of the twelve monocotyledon species examined. The amount of polygalacturonase ranged from 6.3 units/g in lily to 201 units/g in Johnsongrass. The C_4 species contained much more polygalacturonase than the C_3 species. The enzyme in corn (*Zea mays* L.) pollen was partially purified and characterized. It was optimally active at pH 5.3 and required Ca^{2+} for activity, with an optimum concentration of 0.5 mM. The enzyme was most effective on galacturonans with moderate chain lengths. It released monomeric units from the non-reducing ends of the substrate chains, and thus can be classified as an exopolygalacturonase. The polygalacturonase in the other grass pollens appeared to be similar to that in corn pollen.

Key words: pollen; polygalacturonase; monocotyledons; pollen tube growth

Introduction

The growth of pollen tubes is associated with the presence of numerous vesicles in the apical tip [1]. In some species, the vesicles are produced by restored dictyosome activity accompanying pollen germination [2]. Both pollen germination and tube growth occur rather slowly in these species. An example is *Lilium longiflorum*, with a pollen tube growth rate of 12 μm/min accompanied by the production of 2150 vesicles/min [3].

Pollen tube growth occurs much more rapidly in the *Gramineae* than in *Lilium* [4]. Growth rates of 240 μm/min have been recorded for *Zea mays* [2]. In contrast to *Lilium*, there are few dictyosomes in extending grass pollen tubes. Heslop-Harrison [5] has proposed that the main dictyosome activity in grasses occurs during pollen maturation and not during pollen tube growth. The grass pollen grain at the time of dispersal contains numerous storage bodies similar in appearance to the vesicles in the growing tip of *Lilium*. Heslop-Harrison [4] has named these bodies polysaccharide particles based on their high content of polysaccharides.

The principal component of the polysaccharide particles in grass pollens [2] and the vesicles of other species is pectic in nature [6,7]. During pollen tube growth, these particles fuse with the wall in the extending regions and contribute precursor materials [2]. Because pectin is a primary component of the cell wall, growth of the pollen tube may represent rearrangement of pectin molecules from the particles to form new walls. We are interested in characterizing the enzymes that are involved in pectin metabolism in pollen and pollen tubes. This paper reports studies of polygalacturonase in pollens from grasses.

Methods

Most of the studies were conducted with corn (*Zea mays* L.) pollen. Tassels from field-grown plants, Pioneer Hybrid X304C, were harvested, placed in water, and transferred to the laboratory. Pollen was collected from newly dehiscing anthers, weighed, frozen in liquid N_2 and stored at −80 °C. Other pollens were collected locally or purchased from Greer Laboratories Incorporated, Lenoir, North Carolina.

0168-9452/89/$03.50

Enzyme extracts were prepared by suspending 1.0 g of pollen in 10 ml of salt solution containing 10 mM $CaCl_2$ and 0.15 M NaCl. The pollen grains were disrupted by grinding with a mortar and pestle followed by ultrasonication for 1 min. All of the steps were conducted in the cold. The homogenate was adjusted to pH 5.0, stirred for 30 min, and centrifuged at 20 000 × *g* for 15 min. The pellet was re-extracted with 5 ml of salt solution as described above. The supernatant solutions were combined, ultrafiltered to 5 ml using a PM-10 membrane (Amicon Corporation), and dialyzed against 4 l of a solution containing 5 mM $CaCl_2$ and 0.15 M NaCl for 16 h.

Purification of corn pollen polygalacturonase

Corn pollen (5.6 g) was extracted with 50 ml of a solution containing 10 mM $CaCl_2$ and 0.15 M NaCl as described above. The pellet was washed with 25 ml of this salt solution and the extracts were combined. The crude extract was dialyzed, concentrated by ultrafiltration to 10 ml and applied to a 2.5 × 90 cm column of Sephadex G-100 equilibrated with a solution containing 10 mM $CaCl_2$ and 0.15 M NaCl. The column was eluted with this solution at a rate of 0.5 ml/min. The fractions were assayed for polygalacturonase and the fractions containing activity were pooled, ultrafiltered to 5 ml and dialyzed against 20 mM NaCl. One milliliter aliquots were then applied to a Mono S HR 5/5 column (Pharmacia Fine Chemicals) in a Fast Protein Liquid Chromatography (FPLC) system. Buffer A consisted of 20 mM MES (pH 6.0) and buffer B consisted of 20 mM MES (pH 6.0), containing 0.5 M NaCl. The system was programmed for linear segments of 0 – 2 min (0% B) and 2 – 30 min (0 – 100% B) at a flow rate of 1 ml/min. The 1-ml fractions were assayed for polygalacturonase and those containing activity were pooled, ultrafiltered to 2 ml and dialyzed against 20 mM NaCl. This solution was rechromatographed on the Mono S column under the same conditions. The fractions containing polygalacturonase were ultrafiltered to 1 ml and dialyzed against 20 mM NaCl.

Assay of polygalacturonase

The reaction mixture contained 0.1 ml of 0.1 M sodium acetate (pH 5.3), 0.2 ml of water, 0.1 ml of 5 mM $CaCl_2$ and 0.1 ml of enzyme solution. Blanks were prepared by heating duplicate enzyme solutions in boiling water for 5 min. The solutions were allowed to equilibrate at 37 °C and the reactions were initiated by adding 0.5 ml of 1% polygalacturonic acid (pH 5.3). After 30 min at 37 °C , the solutions were analyzed for reducing groups by the arsenomolybdate method [8]. A unit of polygalacturonase is defined as that amount which catalyzes the hydrolysis of 1 μmol glycosidic linkages in 30 min under these conditions.

Polygalacturonase was also assayed viscometrically [13]. The reaction mixture was prepared as described above except that the substrate was pectate and the concentration of Ca^{2+} was decreased to 0.1 mM to prevent gelling of the pectate. Five milliliters of the reaction mixture was placed in an Ostwald viscometer immersed in water at 37 °C and the viscosity was measured at regular intervals. Pectate, polygalacturonic acids, and oligogalacturonides were prepared and analyzed as described earlier [9].

Other methods

Protein was measured by the Bradford method [10] and uronic acids were measured with the hydroxydiphenyl reagent [11].

Results

Purification of corn pollen polygalacturonase

The crude extract of corn pollen contained considerable amounts of soluble polysaccharides including pectin as determined by the hydroxydiphenyl method [11]. These high molecular weight components were separated from the polygalacturonase by gel filtration on Sephadex G-100 as the first step in the purification procedure. The enzyme solution was then chromatographed on the cation exchanger Mono S (Fig. 1). The enzyme recovered from this step was rechro-

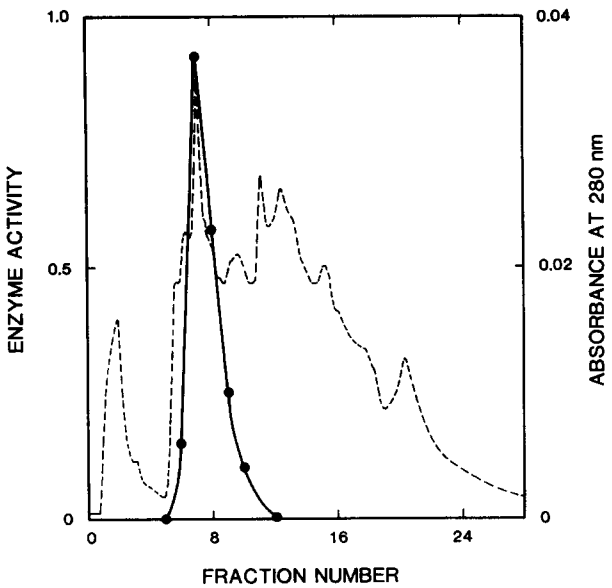

Fig. 1 Chromatography of corn pollen polygalacturonase on Mono S. ●———●, units of enzyme activity; — — —, absorbance at 280 nm.

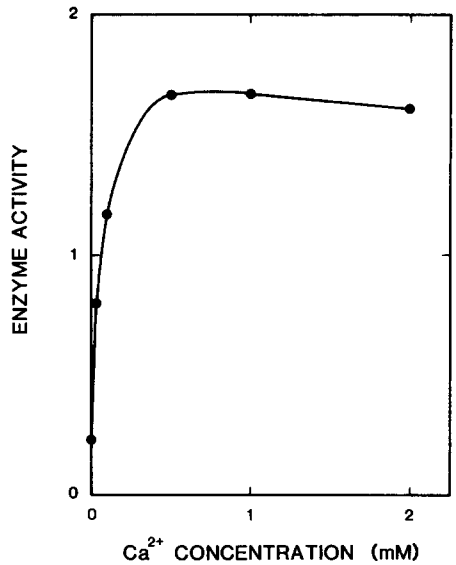

Fig. 2. Effect of Ca^{2+} on the activity of corn pollen polygalacturonase. The enzyme activity is expressed as μmol reducing groups/30 min.

matographed on the Mono S column with a further increase in specific activity and a final purification of 55-fold. A summary of the purification procedure is presented in Table I.

Properties of corn pollen polygalacturonase

Polygalacturonase activity in dialyzed crude extracts of corn pollen was low when assayed in the absence of Ca^{2+}. It was completely abolished by chelating agents such as citrate and ethylenediaminetetraacetic acid (EDTA). Consistent with the properties of polygalacturonases from other plant sources [12], the corn pollen enzyme required Ca^{2+} for maximal activity. Addition of 0.5 mM Ca^{2+} to the reaction mixture activated the purified enzyme 7-fold over the water control (Fig. 2). The enzyme was also activated by Cd^{2+} but to a lesser extent than by Ca^{2+} on an equimolar basis. Other divalent cations were not effective and high concentrations (50 mM) of NaCl were inhibitory.

The molecular weight of the polygalacturonase was determined by gel filtration on a 2.5 × 90 cm column of Sephadex G-100 equilibrated with 20 mM NaCl. From the elution volumes of the protein standards bovine serum albumin

Table I. Summary of the purification of polygalacturonase from corn pollen.

Fraction	Volume (ml)	Protein (mg)	Total act. (units)	Spec. act. (units/mg)	Yield (%)
Crude	68	38.0	335	8.8	—
Sephadex G-100	5	9.2	280	30.4	84
Mono S	2	0.6	227	378.0	68
Second Mono S	1	0.4	192	480.0	57

(BSA), ovalbumin, carbonic anhydrase and cytochrome *c*, the molecular weight of the enzyme was calculated to be 51 000.

The polygalacturonase was active between pH 4.0 and 7.5, with an optimum at pH 5.3. The pH optimum was independent of Ca^{2+} concentration and the degree of polymerization of the substrate. However, the rate of hydrolysis by the enzyme was highly dependent on the chain length of the substrate. Digalacturonic acid was hydrolyzed very slowly. The reaction rate increased with galacturonan chain length to a maximum at a degree of polymerization of about 15 (Fig. 3). The rate of hydrolysis then decreased for larger substrates perhaps due to insolubilization of the larger acidic polymers by Ca^{2+} in the reaction mixture.

While corn pollen polygalacturonase released reducing groups from sodium pectate, it was rather ineffective in degrading this substrate. This was determined by the viscometric assay. The formation of reducing groups was monitored along with the changes in viscosity during the reaction. The reducing groups increased at a linear rate to 1.7% of the glycosidic linkages in pectate after 2 h but the viscosity of the solution decreased only 4.6%. The small effect on the viscosity of pectate is consistent with an exo-cleaving enzyme [13].

The product of corn pollen polygalacturonase action on pectate was identified in a reaction mixture incubated for 16 h at 25°C. The solution was treated with 2 vol. of ethanol to precipitate the unhydrolyzed pectate. The supernatant solution was evaporated to dryness, dissolved in water and analyzed by high performance liquid chromatography (HPLC) on a Polyanion SI column as described earlier [9]. A single peak of uronic acid was obtained and it corresponded to a galacturonic acid standard.

The mode of action for corn pollen polygalacturonase was further elucidated by

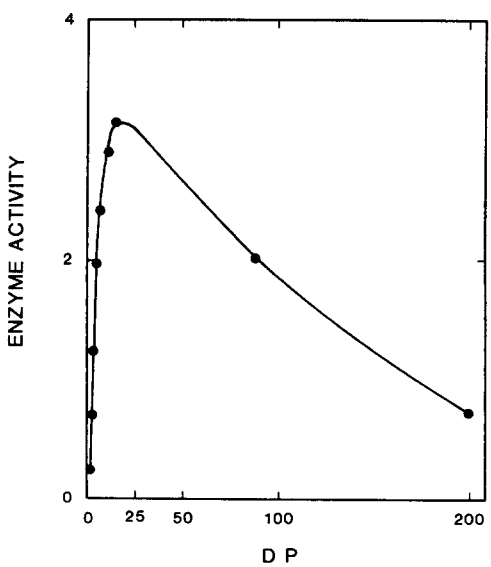

Fig. 3. Effect of substrate size on the activity of corn pollen polygalacturonase. DP, degree of polymerization. The enzyme activity is expressed as μmol reducing groups/30 min.

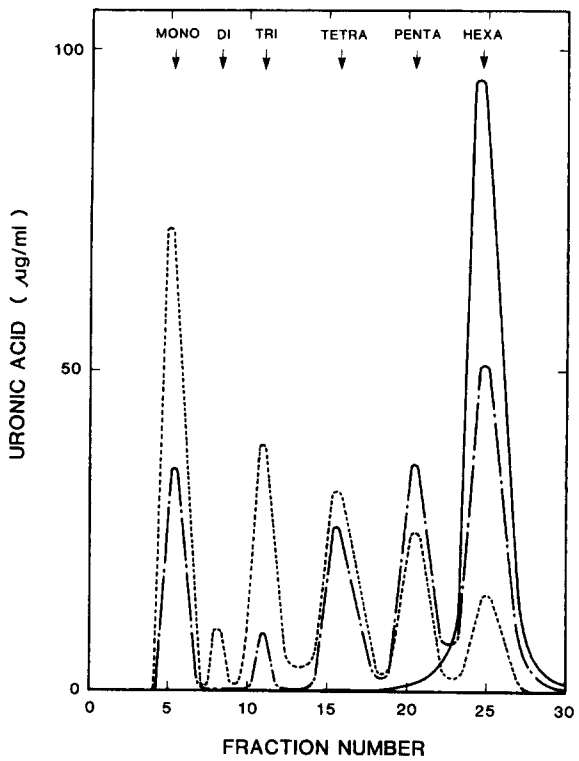

Fig. 4. Hydrolysis of hexagalacturonic acid catalyzed by corn pollen polygalacturonase. The reaction mixture was analyzed for galacturonic acid and oligogalacturonides by HPLC [9] after the reaction periods indicated. ————, 0 time; ——· ——, 1 h; - - - - - - - -, 2 h.

Table II. Concentrations of polygalacturonase in pollens

Common name	Species	Polygalacturonase (units/g)
Lily	*Lilium longiflorum* Thumb.	6.3
Reed canary grass	*Phalaris arundinacea* L.	6.7
Oat	*Avena sativa* L.	7.2
Palm	*Cocos plumosa* L.	8.0
Timothy	*Phleum pratense* L.	8.7
Rye	*Secale cereale* L.	11.3
Wheat	*Triticum aestivum* L.	12.5
Meadow fescue	*Festuca elatior* L.	14.6
Corn	*Zea mays* L.	83.0
Pearl millet	*Pennisetum americanum* L.	87.0
Sorghum	*Sorghum vulgare* L.	105.0
Johnsongrass	*Sorghum halapense* L.	201.0

determining the sequence of products from hexagalacturonic acid. Reaction mixtures of the enzyme and the hexamer were incubated for various times, heated 5 min at 100 °C, and then analyzed by HPLC on a Polyanion SI column. The first detectable products were galacturonic acid and pentagalacturonic acid (Fig. 4). These products increased with time, and gradually tetragalacturonic acid and trigalacturonic acid appeared. As the hexamer was exhausted, the pentamer began to decrease, with accumulation of the smaller oligomers and galacturonic acid. The dimer appeared in the mixtures very slowly. The results confirm that corn pollen polygalacturonase is an exoenzyme that removes monomeric units from the substrate in a stepwise fashion.

Polygalacturonase in other pollens

The extraction and assay conditions established for corn pollen were applied to determine the levels of polygalacturonase in several other monocots (Table II). The values ranged from 6.3 units/g in lily pollen to 201 units/g in Johnsongrass pollen. The C_3 grasses, reed canary, oat, timothy, rye, wheat and fescue, contained slightly more polygalacturonase than the pollen from lily. The pollens from the C_4 species, corn, pearl millet, sorghum and Johnsongrass, contained at least 5 times more polygalacturonase than those from the C_3 species. The amount of polygalacturonase in corn pollen represents about 200 times more than that found in corn seedlings [12].

The crude extracts of all the pollens were examined for the possible presence of endopolygalacturonase by measuring activity by the viscometric assay. Each had a small effect on the viscosity of pectate relative to the rate of release of reducing groups. All of the enzymes were activated by Ca^{2+} and inhibited by chelating agents. The enzymes were optimally active between pH 5.0 and 5.5. Thus it appears that the polygalacturonases in pollens are exoenzymes similar to that in corn pollen.

Discussion

Numerous enzymes have been detected in pollen including those involved in the degradation of some cell wall polysaccharides [14]. The number of reports on pectin-degrading enzymes in pollen has been rather small. There has been a tendency to refer to these enzymes as pectinases [14], presumably because the enzymes were assayed with pectin as the substrate using a viscometric assay. The present study shows that the pectic enzyme in grass pollens is specific for deesterified pectin, i.e., pectate or polygalacturonic acid. Because the polygalacturonase in pollen is an exoenzyme, the viscometric assay would not be suitable or

sensitive for measuring the activity. Furthermore, an adequate level of Ca^{2+} in the reaction mixture is required for the maximal activity of the enzyme to be expressed.

The exopolygalacturonase in pollen appears to be similar to the enzymes found in other plant tissues [12]. In addition to the requirement for Ca^{2+} and specificity for deesterified substrates, pollen polygalacturonase is most effective in hydrolyzing relatively large galacturonans. Digalacturonic acid is particularly resistant to its action.

Exopolygalacturonase in pollen may have a role in the dissolution of the pectin-rich stratum between the cuticle and cellulosic wall of the stigma [15] during pollen tube penetration following pollination. Another possible role for exopolygalacturonase is in the expansion of the pollen tube. The evidence for the involvement of pectin-rich pollen tube vesicles [1,6,7] and pollen polysaccharide particles [2,5] in tube growth suggests a requirement for pectic enzymes in the process. Pollen tube growth is known to respond markedly to exogenous Ca^{2+} [16], and activation of exopolygalacturonase by this cation is consistent with a role for the enzyme in tube growth. But it is unlikely that the pectic reserves in these particles would simply be converted to galacturonic acid by exopolygalacturonase before incorporation into the polymers of the pollen tube. The high rates of pollen tube growth [2] suggest that mobilization and utilization of relatively large blocks of pectin may be involved, although the role of exopolygalacturonase in such a mechanism is not clear.

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